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Note

A New and Efficient Method for Gene Transfer into Mouse FM3A Cells Using Metaphase Chromosomes by Electroporation

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We introduced chromosome-mediated genes into mouse thymidine kinase-deficient FM3A (FM3Atk$^-$) cells, by electroporation. The effects of some parameters on the electric shock-mediated transfection of FM3Atk$^-$ cells were investigated. Gene transfer of mouse L929 metaphase chromosome DNA into FM3Atk$^-$ resulted in a maximum frequency of $(3.0 \pm 0.3) \times 10^{-5}$ at a cell density of 2.0×10^8/ml and chromosome dosage of 5.0×10^7 cell equivalents/ml in a buffer containing 0.25 M mannitol, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM Tris–HCl (pH 7.1). The highest yield of the transformants was obtained at an electric field strength of 1 kV/cm and a capacitance of 35 μF, with a single exponentially decaying pulse at 0°C. The incubation conditions of 60 min at 0°C was optimal for post-shock incubation after electroporation. The tk gene was detected in the transformants by *in situ* hybridization analysis.

Key words: electroporation; gene transfer; chromosome; mouse FM3A; frequency

Cells in suspension are transiently permeabilized upon exposure to some critical electric field strength.[1] This technique, designated as electroporation, is widely available for introduction of exogenous DNAs and other macromolecules into living cells. Electroporation has been proved superior to conventional methods such as calcium phosphate precipitation or microinjection. Since the first application to mouse fibroblasts by Neumann *et al.*[2] it has been widely used in molecular biology and genetics because DNA can easily be transferred into various procaryotic and eukaryotic cells.[3] A high frequency of transfer can be achieved for each cell type, by selecting a series of parameters of pulse amplitude and duration.[3–5] However DNAs thus far used for the gene transfer are mainly plasmids, which are rather small. The electroporation has not been used for chromosomal DNA transfer. Metaphase chromosomes have been transferred to cells by co-precipitation with calcium phosphate.[6–9] In these previous studies, the transfer frequency (number of transformants total viable cells) was only 10^{-6}–10^{-7}. This method is also low in reproducibility and requires a certain skill in handling. Here we report a new electroporation protocol that shows a considerably higher frequency of chromosome-mediated gene transfer.

The thymidine auxotrophic strain, mouse FM3A cells, was cultured as described by Yamauchi *et al.*[9] The metaphase chromosomes containing a thymidine kinase (tk) gene were prepared from mouse L929 cells as described by Lewis *et al.*[8] The suspension of metaphase chromosomes diluted in 80 μl of a electroporation buffer composed of 250 mM mannitol, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM Tris–HCl (pH 7.1) was added to pellets of FM3Atk$^-$ cells (10^5–10^6), and a 50-μl portion of the cell suspension was then placed in a electroporation cuvette (interelectrode distance, 0.1 cm). An exponentially decaying wave pulse (the field strength, 1 kV/cm; the capacitance, 35 μF) was applied to the cuvette with an electroporation apparatus (Electro Gene Transfer Equipment GTE-10, Shimadzu, Japan). After incubating the mixture for 60 min at 0°C (unlike conventional post-shock incubation[10] at 37°C), the mixture was added to 10 ml of ES medium (Nissui Seiyaku Co., Ltd., Japan) with 2% fetal calf serum (FCS), in 10-cm Petri dishes. This sample was then cultured for 12 h at 37°C for the gene expression. The culture was then diluted and overlayed on ES agar plates containing 10% FCS and HAT supplement and incubated for 2–3 weeks until tk$^+$ clones developed.[6]

The electric pulse-mediated DNA transfer into FM3Atk$^-$ cells strongly depends on the initial strength of the electric impulses.[2] As shown in experimental parameter A in Table I, the electric impulse at 1 kV/cm was most effective for the chromosome gene transfer. At field strengths higher than 3 kV/cm, the cells were irreversibly damaged. The presence of divalent cations like Mg^{2+} increased the amount of DNA bound to the cell surface.[11] As shown in experimental parameter B in Table I, the frequency in chromosome gene transfer increased with an increase of the concentration of Mg^{2+} ions in the electroporation buffer, and gave the highest value of $(2.3 \pm 0.2) \times 10^{-6}$ at 0.5 mM of Mg^{2+} ions. The frequency also became higher with an increasing concentration of mannitol in the buffer and gave the maximum

Table I. Chromosome-mediated Gene Transfer into FM3Atk$^-$ Cells by Electroporation

Experimental parameter		Survival (%)	Frequency
A: Electric impulse	0.5 kV/cm	92	$(2.1 \pm 0.2) \times 10^{-6}$
	1.0 kV/cm	80	$(4.7 \pm 0.1) \times 10^{-6}$
	1.5 kV/cm	14	$(3.3 \pm 0.3) \times 10^{-6}$
	2.0 kV/cm	4	$(1.6 \pm 0.1) \times 10^{-6}$
	3.0 kV/cm	0	0
B: Mg^{2+} conc.	0.05 mM	64	$(1.3 \pm 0.1) \times 10^{-6}$
	0.1 mM	62	$(1.6 \pm 0.2) \times 10^{-6}$
	0.2 mM	67	$(1.6 \pm 0.1) \times 10^{-6}$
	0.5 mM	65	$(2.3 \pm 0.2) \times 10^{-6}$
	1.0 mM	64	$(1.8 \pm 0.3) \times 10^{-6}$
C: Mannitol conc.	0.1 M	55	$(2.0 \pm 0.1) \times 10^{-5}$
	0.25 M	38	$(2.7 \pm 0.1) \times 10^{-5}$
	0.5 M	30	$(1.6 \pm 0.2) \times 10^{-5}$
	1.0 M	19	$(7.5 \pm 0.8) \times 10^{-6}$

The standard electroporation was done, using samples containing 10^5–10^6 cells/ml and chromosome of 10^5–10^8 cell equivalents/ml in buffer as described in the text, under electric conditions of a field strength of 1 kV/cm and a capacitance of 35 μF with a single decayed pulse at 0°C. Frequency was presented as the mean ± standard deviation number obtained from triplicate trials.

Table II. Chromosome-mediated Gene Transfer into FM3Atk⁻ Cells by Electroporation

Experimental parameter		Survival %	Survival Fold	Frequency
A: Cell density	5.0×10^5 cells/ml	1		0
	5.0×10^6 cells/ml	14		0
	5.0×10^7 cells/ml	55		$(2.7 \pm 0.5) \times 10^{-6}$
	5.0×10^8 cells/ml	57		$(1.3 \pm 0.2) \times 10^{-5}$
B: Chromosome conc.	1.1×10^5 cell equiv./ml	64		$(1.3 \pm 0.2) \times 10^{-6}$
	1.1×10^6 cell equiv./ml	74		$(2.9 \pm 0.7) \times 10^{-6}$
	1.1×10^7 cell equiv./ml	80		$(7.2 \pm 0.6) \times 10^{-6}$
	1.1×10^8 cell equiv./ml	81		$(1.6 + 0.03) \times 10^{-5}$
C: Post-shock incubation	0°C 0 min	75		$(1.8 \pm 0.2) \cdot 10^{-5}$
	0°C 10 min	62		$(2.1 \pm 0.1) \cdot 10^{-5}$
	0°C 30 min	50		$(2.4 \pm 0.2) \cdot 10^{-5}$
	0°C 60 min	47		$(3.0 \pm 0.3) \cdot 10^{-5}$
	0°C 120 min	41		$(2.3 \pm 0.1) \cdot 10^{-5}$
	20°C 60 min	38		$(1.7 \pm 0.1) \cdot 10^{-5}$
	37°C 60 min	32		$(1.1 \pm 0.2) \cdot 10^{-5}$
D: Gene expression time	0 h		1.0	$(7.0 \pm 7.0) \cdot 10^{-8}$
	2 h		1.2	$(2.8 \pm 1.4) \cdot 10^{-7}$
	6 h		1.8	$(5.6 \pm 0.7) \cdot 10^{-7}$
	12 h		3.0	$(1.2 \pm 0.2) \cdot 10^{-6}$
	24 h		4.1	$(8.4 \pm 0.7) \cdot 10^{-7}$
	36 h		2.5	$(2.1 \pm 0.1) \times 10^{-7}$

The standard electroporation procedure was the same as in Table I.

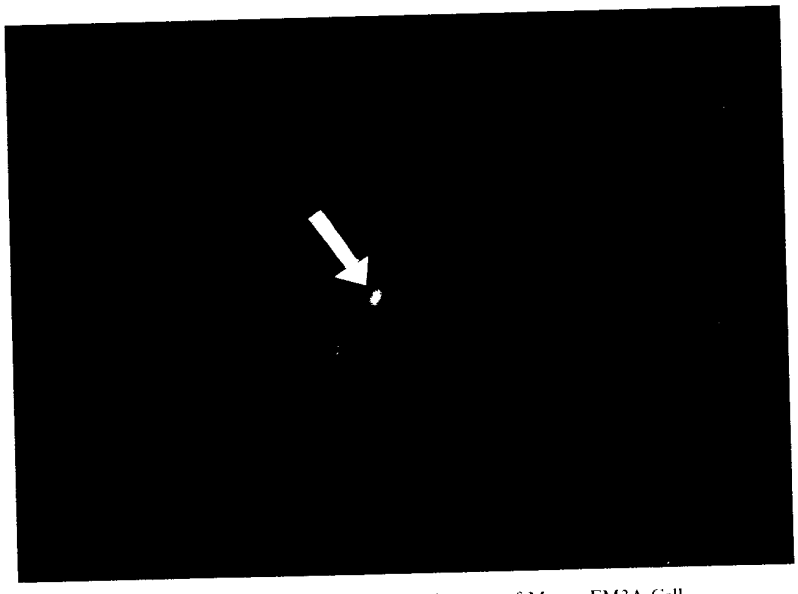

Fig. Fluorescence Photomicrograph of Metaphase Chromosomes in a Transformant of Mouse FM3A Cell.

FM3Atk⁻ chromosome DNA was hybridized with a probe of pTKS plasmid DNA containing tk⁺ gene and stained with propidium iodide (Sigma, P4170).[12,13] The arrow indicates the fluorescence of tk gene integrated in the FM3Atk⁻ cell centromere.

value of $(1.6 \pm 0.2) \times 10^{-5}$ at 0.5 M (Parameter C in Table I). Towards the higher cell density the yield of the transformants increased, but the frequency was the highest at 5.0×10^7 cells/ml. Gene transfer into cells was not detected at cell densities lower than 5×10^6 ml (Parameter A in Table II). The survival rate was reduced with the decrease of the cell density. This result indicates that the cells are irreversibly damaged with the increased collision of pulse to cells at lower cell density. It is important to analyze the relationship between dosage of chromosome and the frequency of gene transfer. The number of chromosome was expressed as cell equivalents per milliliter (Parameter B in Table II). The frequency increased markedly with an increase of the concentration of L929 chromosome up to 1.1×10^8 cell equivalents/ml. When the cells were held, after exposure to the electric shock, at 0°C for 60 min the surviving fraction was slightly decreased, while the transformation frequency increased moderately (Parameter C in Table II). We also investigated the effects of incubation time for gene expression at 37°C, following post-shock incubation after electroporation, on the transfection frequency (Parameter D in Table II). The frequency increased with prolongation of incubation time and reached the maximum at 12 h. Cell survival also increased gradually during the incubation and reached a peak at 24 h. At

36h, however, the surviving fraction was rather reduced, due to the lack of nutrients.

To detect physically the transgenome, fluorescence *in situ* hybridization[12,13] was next done. Using plasmid pTKS[14] (8.0 kbp) containing the tk gene as a probe, the tk genes were hybridized to the chromosomal DNA from transformants (see Fig.). Fraction of the hybridized cells was found to be about 5% among $\sim 10^2$ transformants examined. These data indicate that the transformant contains L929 chromosomal DNA integrated in the host chromosome. It was found that the progeny cells obtained by successive culture of the transformants also contain L929 chromosomal DNA in the host chromosome, by *in situ* hybridization analysis (data not shown). It is not clear if rearrangements, particularly interstitial deletions, occur in the transgenomes taken up recipient cells. Willecke *et al.*[15] suggest that transfer fragmentation of the transgenome can occur during, or before, its integration. Yamauchi *et al.*[9] reported that the transformant may contain human HeLa chromosome DNA integrated in the host chromosome that was as small as several hundred kilobase pairs in size, by analyzing DNA bands containing human Alu sequences. McBride *et al.* roughly estimated that the size of the transgenome was no more than 0.25–0.3% of the donor genome.[6,16]

Yamauchi *et al.*[9] also showed that the frequency of transfer of HeLa chromosome into FM3Atk$^-$ cells was only 10^{-6}–10^{-7} per cell by co-precipitation of mitotic chromosome with calcium phosphate. Our experiments on mouse L929 chromosome transfer into FM3Atk$^-$ cells resulted in the order of 10^{-5} in frequency and provide guidelines for the identification of conditions suitable for electrically mediated chromosomal gene transfer. At least the initial field strength of 1.0 kV/cm and the capacitance of 35 μF may be applied at 0°C using the pulsing buffer containing 0.5 mM $MgCl_2$ and 0.5 M mannitol. Electroporation will be generally applicable for chromosome gene transfer into mammalian cells. Chromosomes of HeLa cells were indeed electrotransferred into FM3A cells, at the efficiency equal to that of L929 strain (unpublished results).

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